REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
28-12-2007	Final Report	1-Oct-2005 - 30-Sep-2007

4. TITLE AND SUBTITLE
Final Progress Report for: Understanding Factors Influencing The Propagation of Prions

5a. CONTRACT NUMBER
W911NF-05-1-0505

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
5D10S1

6. AUTHORS
Susan W. Liebman

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
University of Illinois - Chicago
Board of Trustees of the University of Illinois
809 South Marshield Avenue
Chicago, IL 60612 -7205

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
48145-LS.1

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for public release; Federal purpose rights

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
Prions are altered conformations of a protein that have gained the ability to convert the normal form of the protein into the prion form. They are an infectious, misfolded and aggregated form of a protein. In mammals, prions are associated with neurodegenerative diseases that can be passed from one organism to another.
Recent evidence has shown that the prion phenomenon is not limited to mammals but extends to yeast. We have investigated if any genes affect the propagation of the yeast prion [PIN+] and found that only two previously known deletions (rnq1 and hsp104) abolish [PIN+] maintenance. However, a deletion of CUE2, a gene implicated in the ubiquitin pathway,

15. SUBJECT TERMS
prion, yeast, species barrier

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Susan Liebman
U	U	U	SAR		19b. TELEPHONE NUMBER 312-996-4662

Report Title

Final Progress Report for: Understanding Factors Influencing The Propagation of Prions

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Prions are altered conformations of a protein that have gained the ability to convert the normal form of the protein into the prion form. They are an infectious, misfolded and aggregated form of a protein. In mammals, prions are associated with neurodegenerative diseases that can be passed from one organism to another.
Recent evidence has shown that the prion phenomenon is not limited to mammals but extends to yeast. We have investigated if any genes affect the propagation of the yeast prion [PIN+] and found that only two previously known deletions (rnq1 and hsp104) abolish [PIN+] maintenance. However, a deletion of CUE2, a gene implicated in the ubiquitin pathway, shows an altered [PIN+] phenotype. We are investigating the basis of this difference, which will provide clues to what genes are involved in the morphology of prion aggregates.
We have also investigated various factors that might affect prion transmission across species. We have found that QN rich prions, but not non-QN rich prions and polyglutamine aggregates, enhance the appearance of a foreign prion. Furthermore, we have found that a cellular factor, UBC4, involved in prion appearance might play a role in the prevention of transmission of a prion across species.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Number of Papers published in peer-reviewed journals: 0.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals: 0.00

(c) Presentations

Liebman, SW. (2007) Prion-Prion Interactions in Yeast. XXIII International Conference on Yeast Genetics and Molecular Biology, Melbourne, Australia.

Liebman, SW. (2007) Prions: Vehicles of Protein Conformation Based Inheritance. 2007 Midwest Stress Response and Chaperone Meeting. Northwestern University. Evanston, IL.

Manogaran, AL. and Liebman, SW. (2007) Taking "cues" from Yeast Prions. Post Doctoral Seminar Series. University of Illinois at Chicago. Chicago, IL.

Vishveshwara, N. and Liebman, SW (2006). Crossing the Prion Species Barrier in Yeast. Yeast Genetics and Molecular Biology Meeting. Princeton University. Princeton, NJ.

Number of Presentations: 4.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Number of Manuscripts: 0.00

Number of Inventions:

Graduate Students

NAME	PERCENT_SUPPORTED
Namitha Vishveshwara	0.63
FTE Equivalent:	**0.63**
Total Number:	**1**

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Susan Liebman	0.20	No
FTE Equivalent:	**0.20**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
Sushant Nanavati	0.07
FTE Equivalent:	**0.07**
Total Number:	**1**

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME PERCENT_SUPPORTED

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Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Final Progress Report for: Understanding Factors Influencing The Propagation of Prions.

List of Illustrations and Tables

Statement of problem studied

Specific Aim 1: Find and investigate genes that affect the propagation of the [*PIN*$^+$] prion. Identification of proteins required for prion propagation will provide targets for drugs that could prevent prion propagation.

Specific Aim 2: Investigate the rules governing the species barrier for prion transmission. In order to control the spread of prion disease it will be important to understand the factors that influence infectivity including infectivity across species lines.

Summary of Results

Specific aim 1: Find and investigate genes that affect the propagation of the [*PIN*$^+$] prion.

A screen to isolate genes required for prion maintenance was performed during the grant period. When fused to GFP, Rnq1p forms distinct cytoplasmic foci in [*PIN*$^+$] cells but displays diffuse cytoplasmic fluorescence in [*pin*$^-$] cells. The single-gene deletion strains available in the *MAT**a*** haploid open reading frame deletion collection (Winzeler et al., 1999) were used to isolate genetic deletions that fail to maintain Rnq1p in the prion form. Earlier work showed that a [*PIN*$^+$] prion variant not only existed in the BY4741 library parent strain (Bradley et al., 2002), but also existed in many of the BY4741 deletion derivatives found in the library (Manogaran and Liebman, unpublished). By mating library strains to a *MATalpha* [*pin*$^-$] tester strain carrying a copper inducible RNQ1:GFP plasmid (described in specific aim 1 of the original proposal), diploids can be directly observed for fluorescent aggregates, indicative of the [*PIN*$^+$] variant originating from the library strains. This straightforward method avoids the technical complications brought about by cytoduction of a large number of samples. Therefore, the deletion collection was manually screened in this way for RNQ1 prion aggregates to identify strains that failed to carry this [*PIN*$^+$] library variant ("library" [*PIN*$^+$]). Of the approximately 4800 deletion strains, over 97% of the strains showed some type of punctate fluorescence, indicative of [*PIN*$^+$]. In 65 strains, Rnq1:GFP displayed complete diffuse fluorescence, indicating that these strains are [*pin*$^-$]. Another 52 strains showed a mixture of cells, with approximately half the cells containing diffuse fluorescence and the remaining cells containing foci.

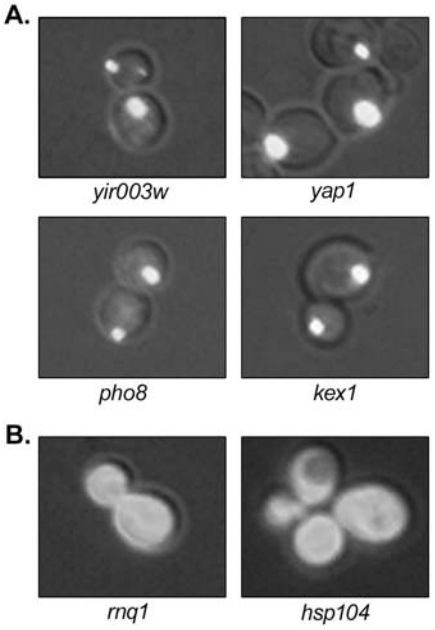

Figure 1. [*PIN*$^+$] aggregates are not maintained in *rnq1* or *hsp104* strains. A) Four strains from the yeast disruption library. In cells carrying the "library" [*PIN*$^+$], staining with RNQ1:GFP results in expected single-dot foci. B) Cytoplasmic diffuse fluorescence in *rnq1* and *hsp104* cells.

While this initial screen identified candidates that failed to contain the "library" [*PIN*$^+$], it does not mean that prion cannot exist without the presence of the candidate gene. To differentiate strains that simply lost the prion from those that failed to maintain [*PIN*$^+$], the strains were cured of all prions by growth on guanidine hydrochloride and the "library" [*PIN*$^+$] was reintroduced via cytoduction (described in specific aim 1 of the original grant proposal). All but two candidates contained aggregates after cytoduction and therefore were able to maintain the introduced "library" [*PIN*$^+$] (Figure 1a). Only *hsp104*Δ and *rnq1*Δ, which have previously been implicated to be involved in [*PIN*$^+$] propagation, failed to maintain [*PIN*$^+$] aggregates (Figure 1b; Derkatch et al., 1997; Sondheimer and Lindquist, 2000; Derkatch et al., 2001).

[*PIN*$^+$] prion variants not only display different conformational characteristics (Bagriantsev and Liebman, 2004), but also exhibit different stabilities. Certain [*PSI*$^+$] variants become unstable in the presence of certain [*PIN*$^+$] variants and differ in the efficiency with which they promote the induction of [*PSI*$^+$] (Bradley and Liebman, 2003). Furthermore, some [*PIN*$^+$] variants are very stable and easily maintained even after many generations, while other forms are less stable and have a propensity to be lost over generations (Manogaran and Liebman, unpublished). The "single-dot" [*PIN*$^+$] variant mostly displays a single focus within the cell similar to the "library" [*PIN*$^+$], whereas "multiple-dot" [*PIN*$^+$] variant cells generally contain numerous smaller foci (Bradley and Liebman, 2003; Figure 2). Introduction of these [*PIN*$^+$] variants into candidate strains lacking any prion showed a consistent profile, single-dot prions exhibited single-dot aggregation profiles, whereas multiple-dot prions displayed multiple-dot profiles. Similar to that observed with the "library" [*PIN*$^+$], only *hsp104Δ* and *rnq1Δ* exhibit diffuse fluorescence, indicating that *HSP104 and RNQ1* are the only two non-essential genes required for the maintenance of the [*PIN*$^+$] prion, regardless of the [*PIN*$^+$] variant used.

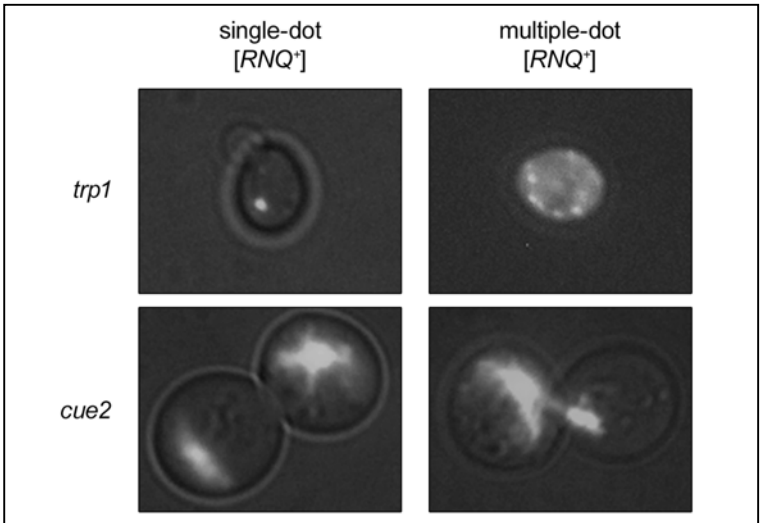

Figure 2. [*PIN*$^+$] aggregates have a different aggregation profile in *cue2* strains. Wild type *CUE2* strains (top; *trp1*) show punctate fluorescence with either single-dot (top left) or multiple-dot (top right) [*PIN*$^+$] variants. Strains containing the *cue2* deletion show tendril-like fluorescence with either single-dot (bottom left) or multiple-dot (bottom right) [*PIN*$^+$].

Upon closer inspection of the original 120 candidates cytoduced with [*PIN*$^+$], one deletion strain exhibited a [*PIN*$^+$] aggregation profile significantly different from that of other strains. The *cue2* deletion strain (Ykl090w) showed large tendril-like aggregates in the presence of the "library" [*PIN*$^+$] or in the presence of either of the two other [*PIN*$^+$] variants, as opposed to the small distinct single or multiple foci observed in other cells.

The *CUE2* gene was initially named due to the presence of a 42 amino acid sequence that shared similarity to a region of Cue1p (Ponting, 2000). The CUE motif, or "coupling of ubiquitin conjugation to ER degradation" motif, was found in Cue1p, which is involved in the recruitment of the ubiquitin-conjugating protein, Ubc7p and results in the degradation of many misfolded ER proteins (Biederer et al., 1997). Over 20 other CUE domain-containing proteins have been identified with functions outside of ER degradation suggesting that the CUE motif could act under different mechanisms (Ponting, 2000). The Cue2 protein contains two CUE domains that bind monoubiquitin independently *in vitro* and mutation of certain residues can affect the binding affinity of ubiquitin (Shih et al., 2003; Kang et al., 2003). The NMR structure studies of the first CUE domain of Cue2p complexed to ubiquitin reveals a similar structure to another motif called UBA, or Ubiquitin-associated motif. UBA motifs have been implicated in binding ubiquitin (Vadlamudi et al.,

1996; Bertolaet et al., 2001; Chen et al., 2001; Raasi et al., 2005). Numerous reviews have detailed the role monoubiquitinylation plays on protein regulation and how it impacts various cellular processes, including endocytosis and DNA repair (Hicke, 2001; Hicke et al., 2005).

We have begun to use genetic and biochemical assays to characterize the altered aggregation phenotype found in the *cue2* deletion strain. We disrupted *cue2* in a routinely used laboratory strain (74D-694). In the presence of a multiple dot [*PIN*$^+$], the aggregation profile was large and circular, different from the punctate dots observed in wild type strains (figure 3; center and right panels). Interestingly, the aggregates do not have the recognizable tendril-like phenotype associated with the original deletion library *cue2* strain (figure 3; left panel). In order to determine whether the genetic background of 74D-694 may be possibly suppressing the phenotype, we are presently engineering a *cue2* disruption in the deletion library parent strain (BY4741).

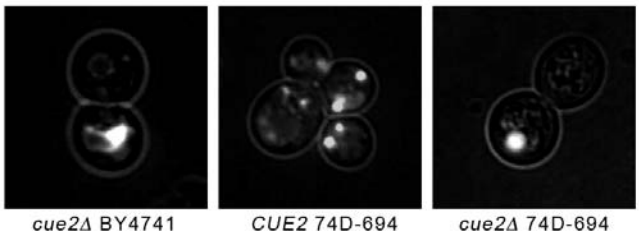

Figure 3. Different genetic backgrounds give different [*PIN*$^+$] aggregation profile when *cue2* is disrupted. Rnq1:GFP aggregates show the spider-like phenotype in *cue2Δ* deletion library strains in the presence of multiple dot [*PIN*$^+$] (BY4741; left). RNQ1:GFP show punctate fluorescence in the wild type 74D-694 strain (center) and large circular aggregates when *cue2* is disrupted (right).

The [*PIN*$^+$] prion has been shown the display a two-level organization, consisting of an SDS-resistant oligomer and a larger SDS-sensitive component (Bagriantsev and

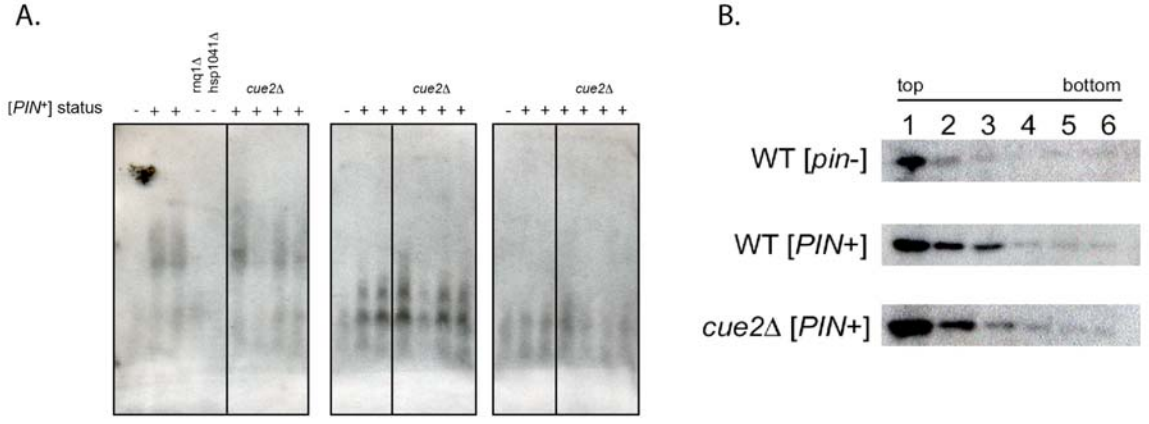

Figure 4. The deletion of *cue2* in [*PIN*$^+$] strains does not appear to impact the secondary structure of Rnq1 aggregates. A. Cell lysates from wild type, *rnq1*Δ, *hsp104*Δ or *cue2*Δ library (as indicated) in the presence of absence of [*PIN*$^+$] were run on SDD-AGE and subjected to Western blot Analysis using an anti-Rnq1 polyclonal antibody (kindly provided by S. Lindquist). Four independent *cue2*Δ lysates were run for comparison. Samples were either incubated at room temperature (left), 60oC (center,) or 100oC (right). B. Lysates of indicated strains were separated by centrifugation on a sucrose density gradient and resolved on 12% SDS-Page and then analyzed for the presence of Rnq1p by Western blot.

Liebman, 2004). It appears that prion variants affect the size of both these two components suggesting that there exists a difference in the structural characteristics of these prions. In order to determine whether *cue2* impacts secondary conformation of the prion, SDS solubilized extracts were subjected to SDD-AGE to resolve SDS-resistant [*PIN+*] oligomers in the original *cue2* deletion library strain. No apparent difference in migration of oligomers was observed in samples, even in the presence of mild heat treatment (Figure 4a). Next, we were interested if the size of the larger [*PIN+*] aggregate is affected by the *cue2* deletion. In wild type cells, the multiple dot [PIN^+] shows penetration into a sucrose gradient. Similar penetration of Rnq1p in [PIN^+] *cue2* strains was observed, indicating that there is no obvious difference in the biochemical profiles between wild type and *cue2* [PIN^+] strains (Figure 4b).

Here, we have shown that appoximately 120 strains of the widely used Winzeler yeast disruption library either did not containg [*PIN+*], or displayed a mixed population of [PIN^+] or [pin^-] cells. When all three variants of the [PIN^+] prion were cytoduced into these deletion strains, only *hsp104Δ* and *rnq1Δ* failed to maintain [PIN^+]. In the examined deletion library strains, a strain containing a deletion of the *cue2* gene caused an altered Rnq1:GFP aggregation profile, without changing the oligomer or aggregation size of the [PIN^+] prion. Our current focus is to determine the basis of this difference, possibly due to the *cue2* deletion, a secondary mutation in the strain background, or a combination of both *cue2Δ* and secondary mutation.

Specific Aim 2: Investigate the species barrier to prion transmission

We had earlier found that the fusion of the prion domain of *P. methanolica* (NM_{PM}) and the C region of *S. cerevisiae* (C_{SC}) goes into a prion form ([CHI^+], which grows on media lacking adenine(-Ade) and are therefore Ade+) only in the presence of [PSI^+] at a frequency of 10^{-4}-10^{-3} vs. a control *sup35* mutant [psi^-] strain which produced no [CHI^+] colonies (Table 1, Figure 5).

[PSI^+]+NM_{PM}-C_{SC}

sup35+ NM_{PM}-C_{SC}

Figure 5: The NM_{PM}-C_{SC} protein is inactivated only in the presence of [PSI^+]. Around 10^7 [PSI^+] or *sup35* mutant cells containing the NM_{PM}-C_{SC} were plated on –Ade –Ura (-Ura to maintain the NM_{PM}-C_{SC} plasmid) media. The chimeric protein was inactivated only in the presence of [PSI^+] and not in a *sup35* mutant.

These Ade+ colonies were confirmed to be [CHI^+] as mentioned previously (The chimeric protein was primarily in the pellet fraction, the phenotype was cytoducible and they contained SDS stable particles). As different variants of [CHI^+] were seen, it suggested that [PSI^+] aggregates were enhancing the *de novo* formation of [CHI^+].

We tested whether the [*CHI*$^+$]s formed in the presence of [*PSI*$^+$] could be maintained in the absence of [*PSI*$^+$]. We cytoduced [*CHI*$^+$][*PSI*$^+$] and [*chi*$^-$][*PSI*$^+$] strains into a *sup35* deletion mutant strain that had a plasmid containing the chimeric protein (NM_{PM}-C_{SC}). This strain was capable of maintaining [*CHI*$^+$] but not [*PSI*$^+$] aggregates. When we used [*CHI*$^+$][*PSI*$^+$] as the donor we were able to find cytoductants that Ade+, which we verified to be [*CHI*$^+$] by checking for SDS stable particles, indicating the [*CHI*$^+$] phenotype is independent of [*PSI*$^+$]. No Ade+ colonies were detected when the donor was [*chi*$^-$][*PSI*$^+$] (data not shown).
To test whether the heterologous prion [*PIN*$^+$] is able to induce the prionization of the chimeric protein, we first cytoduced different [*PIN*$^+$] variants into a sup35 mutant strain. This allowed us to assay for the prion form of the chimeric protein. The chimeric protein converts into a [*CHI*$^+$] state at a rate of around 10^{-5}-10^{-4} (Figure 6, Table1). This prionization occurs in all [*PIN*$^+$] variants but not in a [*pin*$^-$] strain.

Table 1

Strain	**Frequency of appearance of [*CHI*$^+$]**
[*PSI*$^+$]	$6.05 * 10^{-4} \pm 3.85 * 10^{-4}$
sup35 mutant	0
sup35 mutant +High [*PIN*$^+$]	$1.5 * 10^{-4} \pm .63 * 10^{-4}$
[*PSI*$^+$] *Δubc4*	$4.9 * 10^{-3} \pm .95 * 10^{-3}$
sup35 mutant *Δubc4*	0

Table1: Frequencies of formation of [*CHI*$^+$] in different strains. All variants of [*PIN*$^+$] were tested qualitatively by serial dilutions but quantitatively for the high [*PIN*$^+$] variant.

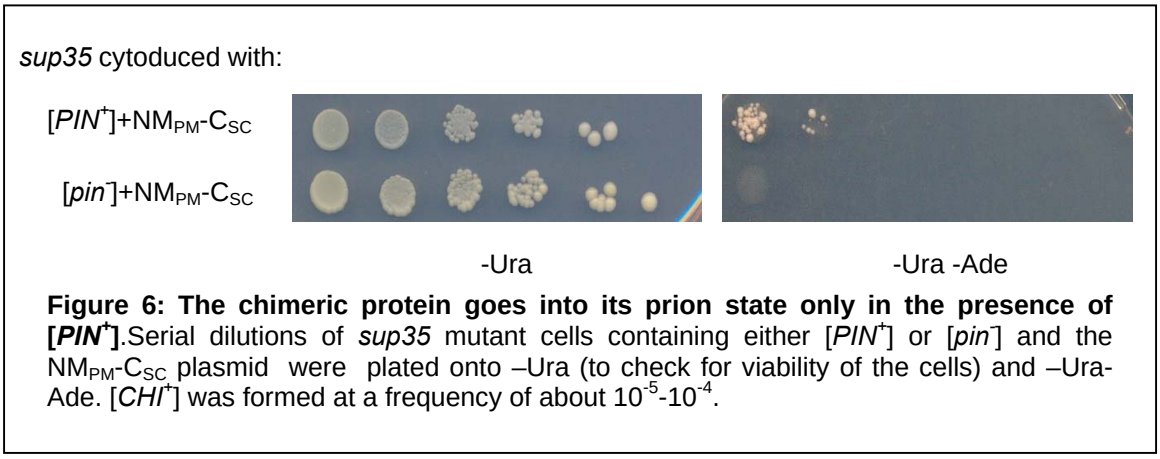

Figure 6: The chimeric protein goes into its prion state only in the presence of [*PIN*$^+$]. Serial dilutions of *sup35* mutant cells containing either [*PIN*$^+$] or [*pin*$^-$] and the NM_{PM}-C_{SC} plasmid were plated onto –Ura (to check for viability of the cells) and –Ura-Ade. [*CHI*$^+$] was formed at a frequency of about 10^{-5}-10^{-4}.

We also tested whether the prion domain of *P. methanolica* can prionize in the presence of the expanded poly glutamine stretch of the Huntington protein. This expanded region of the Huntington protein aggregates in neuron of humans afflicted with the Huntington disease (DiFiglia et al., 1997) and has been shown to aggregate in yeast (Krobitsch and Lindquist, 2000). The *sup35* mutant was transformed with a plasmid containing the first exon of the Huntinton protein which had either a stretch of 25(does not cause the disease) or 103 (disease causing) glutamines fused to GFP. The plasmid containing the stretch of a 103 glutamines aggregated in the *sup35* mutant similar to the wild type strain. However, this non-prion aggregate did not mediate the prionization of the chimeric protein (data not

shown). We then asked whether a prion that is not rich in glutamines and asparagines would increase the appearance of the foreign prion [*CHI*$^+$]. To do this we used the non QN rich HET-s prion from *P. anserina*. The prion domain of HET-s fused to GFP (HET-s(PrD)-GFP) has been shown to propagate as a prion in yeast (Taneja et al., 2007). The *sup35* mutant was transformed with a plasmid containing the HET-s(PrD)-GFP fusion protein under a galactose inducible promoter. At high levels of galactose HET-s(PrD)-GFP is induced into a prion state and maintains this prion state at lower levels of galactose. At continuously low levels of galactose the HET-s(PrD)-GFP fusion does not prionize. We prionized the HET-s(PrD)-GFP fusion in the *sup35* mutant and tested whether [*CHI*$^+$] is formed at a level higher than when HET-s(PrD)-GFP is in its non-prion, diffuse state. The non QN rich prion fails to induce the chimeric protein from going into its prion form (data not shown). Thus, only the QN rich prions [*PSI*$^+$] and [*PIN*$^+$] induces the prionization of the chimeric protein.

Since the appearance of [*CHI*$^+$] seems to be a *de novo* event, we went on to test cellular factors that are known to affect the appearance of [*PSI*$^+$]. The transient overexpression of the chaperone *SSA1* increases the frequency of *de novo* formation of [*PSI*$^+$] (Chernoff et al. 1999). The transient overexpression of *SSA1* (A plasmid containing *SSA1* under a galactose inducible promoter was grown transiently in galactose to overexpress *SSA1*) did not increase the appearance of [*CHI*$^+$]. We also tested the simultaneous deletion of *SSB1* and *SSB2*, which also increases the appearance of [*PSI*$^+$](Newnam et al. 1999), but the double deletion increased the growth of [*PSI*$^+$] cells on –Ade. As this was our assay (growth on –Ade) for the appearance of [*CHI*$^+$], it would be impossible to distinguish between better growth due to the deletion versus the increased appearance of [*CHI*$^+$]. Similarly, the deletion of *UBP6* (a deubiquitinating enzyme) is known to decrease the appearance of [*PSI*$^+$] (Chernova et al. 2003). However, we found that the deletion decreases growth on –Ade even in the *sup35* mutant. Therefore, it was impossible again to distinguish between poor growth due to the deletion and reduced appearance of [*CHI*$^+$].

The deletion of *UBC4*, which is a ubiquitin conjugating enzyme (Allen et al. 2007) in the ubiquitination pathway, has been shown to increase the *de novo* appearance of [*PSI*$^+$] in the presence and absence of the [*PIN*$^+$] prion. We tested the effect of this deletion on the formation of [*CHI*$^+$] in the presence of [*PSI*$^+$]. The deletion increased the formation of [*CHI*$^+$] in the presence of [*PSI*$^+$] (Figure 7, Table1) but the spontaneous appearance of [*CHI*$^+$] in the mutant strain did not increase (Table1).

Figure 7: *Δubc4* increases the appearance of [*CHI*$^+$] in [*PSI*$^+$] strains. Serial dilutions of [*PSI*$^+$] and [*PSI*$^+$] *Δubc4* transformed with the NM_{PM}-C_{SC} plasmid on –Ura (to check the viability) and –Ura-Ade (to check for the appearance of [CHI+])

Thus, we see that *UBC4*, a cellular factor involved in the appearance of [*PSI*$^{+}$] might also be involved in the controlling of prion transmission across the species barrier.

Here we have shown that QN rich prions are capable of enhancing the *de novo* formation of the foreign prion [*CHI*$^{+}$]. A non QN rich prion and a non-prion aggregate are not capable of inducing the prionization of [*CHI*$^{+}$]. In addition we have seen that deletion of one cellular factor (*UBC4*) involved in the appearance of [*PSI*$^{+}$], also enhances the appearance of the foreign prion [*CHI*$^{+}$]. This deletion does not enhance the spontaneous appearance of [*CHI*$^{+}$] but only enhances [*CHI*$^{+}$] appearance in the presence of [*PSI*$^{+}$]. It appears that *UBC4* is involved in the transmission of the prion state between yeast species. The yeast system might help in elucidating more factors that are involved in the prevention of prion transmission from one species to another.

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